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(54) Title: METHOD FOR DETERMINING PLASMINOGEN ACTIVATOR INHIBITOR

(57) Abstract

A method is provided for determining active plasminogen activator inhibitor – Type 1 (PAI–1) in a biological fluid, the method comprising the steps of (i) providing a sample of a biological fluid; and (ii) measuring the amount of PAI–1/multimeric vitronectin complex in the sample to determine active PAI–1 in the sample. A kit for carrying out the method is also provided.

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METHOD FOR DETERMINING PLASMINOGEN ACTIVATOR INHIBITOR

Field of the Invention

This invention relates to the determination of the level of active plasminogen activator inhibitor Type 1 in samples such as biological fluids.

Background of the Invention

In order to ensure an adequate blood supply to various organs, the mammalian body is equipped with two systems, a coagulation system and a fibrinolytic system. The coagulation system functions to stop bleeding and protect the mammal from blood loss. The fibrinolytic system functions primarily to dissolve blood clots. The two systems are normally in equilibrium and the enzymes involved in both systems are under control at multiple levels.

The key enzyme of the fibrinolytic system is plasmin, which digests the fibrin threads of a fibrin blood clot. Plasmin is formed when its precursor protein, plasminogen, is activated by a plasminogen activator. Plasminogen activators are typical serine proteases and four different plasminogen activator (PA) systems are recognized; (a) factor XII-dependent system, (b) streptokinase (isolated from Streptococci), (c) tissue plasminogen activator (tPA) and (d) urinary plasminogen activator (urokinase or uPA). In humans, only tPA and uPA have physiological importance, tPA being the main fibrinolytic enzyme in the circulation.

The plasminogen activating activity of tPA and uPA is inhibited by several plasminogen activator inhibitors (PAI). Four types of PAI have been described: (a) endothelial-type inhibitor (called Plasminogen Activator Inhibitor Type 1 or PAI-1); (b) placental inhibitor (called Plasminogen Activator Inhibitor Type 2 or PAI-2); (c) heparin-dependent inhibitor (Plasminogen Activator Inhibitor Type 3); and (d) the protease nexin (Plasminogen Activator Inhibitor Type 4) (Urden et al., (1987), Thromb. Haemost., v. 57, pp. 29-34; Francis et al., (1988), Am. Heart J., v. 115, pp. 776-780; and Kurnik, (1995), Circulation, v. 91, pp. 1341-1346).

Apart from PAI-2 which plays a role in pregnant women, PAI-1 appears to be the only PAI which is important in humans. It is the primary inhibitor of plasminogen activators in the circulation and is secreted into plasma mainly by endothelial cells and the α granules of the platelets. PAI-1 has a great affinity for its target enzymes and, upon binding, both PAI-1 and the plasminogen activator in the formed complex (PAI-1/tPA or PAI-1/uPA) are inactivated. Upon its release from the endothelial cells into the circulation, tPA is quickly captured by PAI-1 and loses activity (more than 95% of tPA in the blood is bound to PAI-1) (Lijnen et al., (1991), J. Biol. Chem., v. 266, pp. 4041-4044).

Previous studies have described the presence of several conformational and functional forms of PAI-1. More than 95% of the total PAI-1 in circulation in humans is found in the platelets, as latent PAI-1. On platelet activation, the latent PAI-1 undergoes a conformational change and is released into the circulation as active PAI-1. The non-platelet PAI-1 in the circulation exists mainly in two forms: inactive PAI-1 or PAI-1 bound to its target enzymes (about 40% of total non-platelet circulating PAI-1) and active PAI-1 or PAI-1 bound to the plasma protein, vitronectin (about 60% of total non-platelet circulating PAI-1) (Wagner et al., (1989), J. Clin. Invest., v. 84, pp. 647-655). Circulating complexes of PAI-1 with its target enzymes are largely PAI-1/tPA, with only a minute amount of PAI-1/uPA complex.

Like PAI-1, vitronectin can exist in several conformational states. Platelet vitronectin is present in both monomeric and multimeric forms, whereas plasma vitronectin is reportedly monomeric (Seiffert (1997), J. Biol. Chem., v. 272, p. 9971). If plasma vitronectin is exposed to denaturing agents, multimeric vitronectin is formed, which has exposed epitopes not present in monomeric vitronectin. It has been shown that active PAI-1 binds to multimeric vitronectin with higher affinity than to monomeric vitronectin and that active PAI-1 isolated from plasma is predominantly complexed with a high molecular weight form of vitronectin (Lawrence t al., (1997), J. Biol. Chem., v. 272, p. 7676). Other studies have, however, reported that both monomeric and multimeric vitronectin bind to PAI-1 and, as noted by Lawrence (supra),

the nature of the interaction of PAI-1 and vitronectin remains the subject of considerable debate.

Numerous clinical reports have documented that failure of the endogenous fibrinolytic capacity is attributable to an increase in serum PAI-1 activity. Stringer et al., (1994), Arterioscler. Thromb., v. 14, pp. 1452-1458, reported that PAI-1 is released at high concentration from activated platelets and is retained within the thrombus by binding to fibrin, resulting in inhibition of local tPA-mediated clot-lysis. Furthermore, the administration of monoclonal antibodies that block the inhibitory activity of PAI-1 reduced clot lysis resistance. In patients with coronary artery disease (CAD), Hamsten et al., (1985), N. Eng. J. Med., v. 313, pp. 1557-1563, have documented that in young survivors of acute myocardial infarction (AMI), an elevated plasma level of PAI-1 up to 3 years after the event was correlated to a higher rate of reinfarction. Since this initial report, several other investigators have confirmed these observations.

The plasma active PAI-1 level was also investigated, and reported elevated, during the acute coronary thrombotic events. Furthermore, in patients with AMI, the plasma level of PAI-1 was correlated with the capacity to lyse a coronary thrombus. In patients who fail to have restored coronary blood flow, as evident by coronary angiography (determined by angiography 24 hr-1 week after AMI) or by the development of a Q-wave on the ECG, a high plasma level of PAI-1 was documented (Sakamoto et al., (1992), Am. J. Cardiol., v. 70, pp. 271-276 and Ogava, (1993), Cardiol., v. 41, pp. 201-208). From the several studies reported, it can be concluded that in patients with CAD, a high plasma level of PAI-1 is associated with a high risk for developing acute coronary ischemia and that in those who develop an acute event, a high plasma active PAI-1 level is associated with an ominous outcome.

To further establish the role of a balanced equilibrium state between tPA and PAI-1 activities in native fibrinolysis, several clinical trials have investigated patient outcome in artificially induced endothelial dysfunction. In patients who were subjected to Percutaneous Transluminal Coronary

Angioplasty (PTCA), the incidence of acute coronary events in the post-PTCA period was correlated with high plasma levels of active PAI-1 around the time of PTCA according to several reports. The incidence of coronary re-stenosis was also investigated and correlated to the levels of plasma PAI-1 (Hara et al., (1995), Cardiology, v. 86, pp. 407-410 and Sakata, (1996), Am. Heart J., v. 131, pp. 1-6).

A further confirmation of the role of PAI-1 in clot lysis was investigated by artificially inhibiting the activity of PAI-1 by either pharmaceuticals or monoclonal antibodies. Levi et al., (1992), Circulation, v. 85, pp. 305-12, have reported that by inhibiting PAI-1 activity through using monoclonal antibodies (Mab), native tPA could lyse a clot. By using N-acetyltetradecapeptide corresponding to the P_1-P_{14} aminoacid sequence of the PAI-1 to inactivate active PAI-1 and enhance fibrinolysis. Eitzman et al., (1995), J. Clin. Invest., v. 95, pp. 2416-2420, reported that the activity of circulatory PAI-1 decreased, although antigen level did not and that native tPA was more effective in dissolving the clot. Ohtani et al., Eur. J. Pharmac., v. 197, pp. 151-156, developed a novel inhibitor of PAI-1, (a butadiene derivative called T-686), that has been shown to inhibit thrombosis in two experimental thrombosis models in rats without affecting bleeding time. Friederich, (1997), Circulation, v. 96, pp. 916-921, showed that neutralization of plasma PAI-1 activity by a low molecular weight inhibitor (XR5118) enhances clot lysis and reduces clot growth in a rabbit thrombosis model.

A number of these studies (for example, Eitzman et al., (1995), J. Clin. Invest., v. 95, pp. 2416-2420) indicate the importance of measuring active PAI-1, which was seen to fluctuate while the total level of PAI-1, as determined by immunoassay, remains stable.

The role of active PAI-1 in clot lysis and its relevance in a number of disease states is well established. The availability of an accurate and reliable method to determine the plasma level of active PAI-1 is therefore of great clinical importance.

Previously described methods for determining the level of circulating active PAI-1 have been of two main types, functional or immunological.

Several direct and indirect functional methods to quantify the fibrinolytic inhibition capacity of biological samples have been described. (Verheijen et al., U.S. Patent No. 4,563,420; Pussard et al., U.S. Patent No. 5,472,851; Sasamata et al., U.S. Patent No. 5,102,787). The most commonly used method, Verheijen et al., (1985), Thromb. Res., v. 39, pp. 281-8, measures inhibition of tPA activity, which is primarily due to PAI-1 activity, through the hydrolysis of either a tPA-specific substrate or a plasmin-specific substrate, plasmin having been produced by the action of tPA upon plasminogen. This hydrolysis results in either a measurable chromogenic change or in the breakdown of a fibrin film resulting in measurable clot lysis.

The European Committee of Fibrinolysis evaluated the various functional methods available for measuring tPA inhibition in a multicentre study and concluded that they have limited accuracy, Gram et al., (1993), Thrombosis and Haemostasis, v. 70, pp. 852-857. The main drawbacks of these methods are the presence of a partitioning step of the plasma euglobulins, the non-standardization of the incubation conditions, and of the form and amount of tPA to be utilized and the indirectness of measurements. Also, some of these methods discount the role of plasmin inhibitor activities in the test samples. Another problem encountered in methods of measuring inhibition of tPA functionally is the fact that the activities of both tPA and PAI-1 are unstable and decrease gradually after sample collection. In blood with high PAI-1 levels, the tPA activity can decrease by 50% in about one minute.

In order to avoid the problems encountered with functional assay methods for measuring active PAI-1, several immunoassay methods have been developed. The simplest assays employ an antibody to PAI-1 in a conventional immunoassay (for example, U.S. Patents Nos. 5,422,245 and 5,629,160). Methods have also been described for measuring active PAI-1 by a two-step procedure: the sample under investigation is divided into two portions and a saturating amount of tPA is added to one portion. The level of PAI-1/tPA complex is then measured in both portions. The difference in the measured amount of the PAI-1/tPA complex between the two portions represents the amount of free or active PAI-1.

Variations on this method have been described, for example, by Amiral et al., (1988), Thrombosis Research, Supplement VIII, pp. 99-113; Sakata et al., U.S. Patent No. 5,352,583; Niewenhuizen et al., (1995), Blood Coagul. & Fibrinolysis, v. 6, pp. 520-6, and in U.S. Patent No. 5,352,583.

Utilising pairs of antibodies specific for different parts of the PAI-1/tPA complex in the above-described two-step procedure did provide a more reliable determination of active PAI-1 than the earlier functional assays. There are, nevertheless, problems with the assay based on measuring total PAI-1/tPA complex before and after adding exogenous tPA. For example, special instrumentation and techniques are required to arrest further in vitro binding of tPA to PAI-1. Sample collection is complicated by the need for acidification to prevent any unintended in vitro interaction between tPA and PAI-1 and problems arise from the non-standardisation of the conditions for tPA/PAI-1 binding and of tPA preparations themselves.

Many of these methods are also time consuming and technically demanding, limiting their value in the clinical laboratory.

Methods have been described for measuring complexes of PAI-1 and vitronectin in platelets. For example, Preissner et al., (1989), Blood, v. 74, pp. 1989-1996 used an immunoassay employing anti-PAI-1 and anti-vitronectin antibodies and found evidence of PAI-1/vitronectin complexes in platelets. In contrast, however, Lang et al., (1996), J. Biol. Chem., v. 271, pp. 2754-2761 and Nordenhem et al., (1997), Scand. J. Clin. Invest., v. 57, p. 453, used a similar assay and did not detect such complexes in platelets, casting doubt on the efficacy of such an assay. Nordenhem et al. also noted that the described method was not applicable to plasma, due to interference by the high level of vitronectin in plasma.

There remains a need for improved methods of determining the level of active PAI-1 in circulation.

Summary of the Invention

The present invention provides a new method for measuring the level of active PAI-1 in a biological fluid, such as whole blood, plasma or serum.

The method of the invention determines the level of active PAI-1 in circulation by determining the amount of PAI-1 complexed to multimeric vitronectin.

The present invention provides an improved method for determining active PAI-1. The method is much less cumbersome than methods involving comparison of PAI-1/tPA complex levels with and without addition of exogenous tPA. The present method, which measures active PAI-1 directly, as the stable PAI-1/multimeric vitronectin complex, is also less subject to interference from uncontrolled factors such as inconsistencies and artifacts of tPA binding than previously described methods for determining plasma active PAI-1.

In accordance with one embodiment of the invention, a method for determining active plasminogen activator inhibitor-Type I (PAI-1) in a biological fluid comprises the steps:

(i) providing a sample of a biological fluid; and

(ii) measuring the amount of PAI-1/multimeric vitronectin complex in the sample to determine active PAI-1 in the sample.

The biological fluid to be assayed may be selected from the group consisting of whole blood, plasma, serum, saliva, amniotic fluid, cerebrospinal fluid, tissue extract or urine.

In accordance with a further embodiment, a kit for determining active PAI-1 in a biological fluid comprises:

(a) a first antibody which binds selectively to PAI-1; and

(b) a labelled second antibody which binds selectively to multimeric vitronectin.

Detailed Description of the Invention

The present invention provides a method for determining active PAI-1 in a biological fluid by determining the amount of PAI-1/multimeric vitronectin complex present in the fluid.

Any detection reagent or detection system which detects and determines the circulating PAI-1/multimeric vitronectin complex may be employed.

The term "antibody", as used herein and if not otherwise specified, includes a polyclonal antibody, a monoclonal antibody, a single chain antibody and antibody fragments such as Fab fragments.

As used herein, an antibody is said to "bind selectively" to a target molecule if the antibody recognises and binds the target molecule but does not substantially recognise and bind other molecules present in a sample containing target molecules.

As used herein, an antibody is said to "bind selectively to multimeric vitronectin" if the antibody recognises and binds multimeric vitronectin but does not substantially recognise and bind other molecules, including monomeric vitronectin, present in a sample.

As used herein, "multimeric vitronectin" means a polymer of monomeric vitronectin that occurs naturally in plasma and contains two to four monomeric units of vitronectin.

"Denatured vitronectin" is a multimeric form of vitronectin formed in vitro when vitronectin is exposed to denaturing conditions; it contains more than four monomeric units of vitronectin.

In accordance with one embodiment of the invention, a sample of a biological fluid is contacted with a first antibody which binds selectively to PAI-1 in the sample to form a complex. This first antibody binds to both active and inactive PAI-1. The sample is then contacted with a second antibody which binds selectively to multimeric vitronectin. The second antibody carries a label which may be a directly detectable label or may be a component of a signal-generating system. The second antibody binds to the active PAI-1 (i.e. PAI-1/multimeric vitronectin complex)/first antibody complex. The resulting complex is separated from the reaction mixture and the second antibody bound to the complex is determined. Detection and determination of the second antibody label or the signal generated by the signal-generating system, compared with suitable calibration standards, permits measurement

of the amount of PAI-1/multimeric vitronectin complex present in the sample and hence determination of active PAI-1 in the sample.

In accordance with a further embodiment, the sample is contacted with a first antibody which binds selectively to multimeric vitronectin and does not bind substantially to monomeric vitronectin. The first antibody carries a detectable label or a component of a signal-generating system. The sample is then contacted with a second antibody which binds selectively to PAI-1. Determination of the PAI-1/multimeric vitronectin complex, and of active PAI-1, is as described above.

The first and second antibodies may be added separately in a two-step procedure or may be added simultaneously.

Active PAI-1 may be determined as PAI-1/multimeric vitronectin complex by the method of the invention in a biological fluid such as whole blood, plasma, serum, urine, saliva, cerebrospinal fluid, amniotic fluid or a tissue extract.

The biological fluid is preferably whole blood, plasma or serum. When blood is collected for assay of active PAI-1 in whole blood, serum or plasma, care must be taken to avoid platelet activation, for example by using citrate as anticoagulant or by employing special blood collection tubes which promote platelet stabilisation and avoid platelet activation during blood collection; examples of suitable commercially available tubes are Stabilyte™ Blood Collection tubes, available from American Diagnostica Inc., and Becton Dickinson tubes, Catalog No. 6457.

The anti-PAI-1 antibodies used in the methods of the invention should be able to recognise PAI-1 when it is bound to multimeric vitronectin. They should therefore be directed against PAI-1 epitopes which remain exposed in the active PAI-1/vitronectin complex.

The anti-multimeric vitronectin antibodies used should recognise multimeric but not monomeric vitronectin. They should therefore be directed against epitopes exposed in multimeric vitronectin but not accessible in monomeric vitronectin. It is believed that the unique epitopes exposed in d natured vitronectin will also be present in the multimeric vitronectin of the

active PAI-1/multimeric vitronectin complex. Antibodies against denatured vitronectin but which do not recognise monomeric vitronectin may therefor be used in the methods of the invention.

The antibodies used may be monoclonal or polyclonal and may be prepared by conventional techniques or obtained from commercial sources.

Anti-PAI-1 antibodies of suitable binding specificity are obtainable, for example, from American Diagnostics, Greenwich, Connecticut, U.S.A. (anti-PAI-1 monoclonal antibody #3780) or Biopool International, Ventura, California, U.S.A. (anti-PAI-1 monoclonal antibody #214101).

Anti-PAI-1 antibodies and anti-multimeric vitronectin antibodies may be prepared by conventional methods.

Either monoclonal or polyclonal antibodies with the desired binding specificity may be used in the methods of the invention. Any of the first, second or third antibodies may be a monoclonal or a polyclonal antibody. It is preferable to use monoclonal antibodies against PAI-1 and multimeric vitronectin.

Polyclonal antibodies suitable for use in the methods of the invention may be developed against PAI-1 and/or multimeric vitronectin in animals such as guinea pigs, rabbits, horses, sheep or goats, which have been immunized with purified PAI-1 or multimeric vitronectin. PAI-1 protein may be purified as described by Gils et al., (1996), Biochem., v. 35, p. 7474, or obtained commercially, for example from Molecular Innovations, Royal Oak, MI or American Diagnostica, Greenwich, CT. Multimeric vitronectin may be prepared, for example, as described by Mosher et al., (1993), J. Biol. Chem., v. 268, p. 24838.

Specific protocols for the production of polyclonal antibodies are well known in the art. Briefly, the method comprises the following steps; (a) administering the selected antigen to an animal in an amount sufficient to induce the production of antibodies; (b) collecting the antisera containing said antibodies from the immunized animal; and (c) recovering the antibodies from the antis ra. In order to increase the immunogenecity of the antigens, various adjuvants may be used, depending on the host species, including Freund's

adjuvant (complete and incomplete), aluminum hydroxide, surface-active substances such as lysolecithin, polyanions, emulsions of oil and keyhole limpet hemocyanins.

Monoclonal anti-PAI-1 or anti-multimeric vitronectin antibodies may also be produced by methods well known in the art. Briefly, the purified protein is injected in Freund's adjuvant into mice over a suitable period of time, spleen cells are harvested and these are fused with a permanently growing myeloma partner and the resultant hybridomas are screened to identify cells producing the desired antibody with the required binding selectivity. Suitable methods for antibody preparation may be found in standard texts such as Antibody Engineering, 2d. edition, Barreback, Ed., Oxford University Press (1995).

Monoclonal antibodies produced by a selected hybridoma clone may be purified by known techniques such as ammonium sulfate fractionation, DEAE cellulose chromatography or affinity chromatography utilizing protein G or A- Sepharose column chromatography, cellulose membranes and agarose and synthetic materials such as cross-linked polysaccharides, polyvinylchloride, polypropylene, polystyrene and the like or their combinations.

Anti-PAI-1 antibodies displaying the desired binding specificity, as described above, may be obtained using screening methods similar to those described by Declerck et al., (1988), Blood, v. 71, p. 220, and anti-multimeric vitronectin antibodies may be screened for desired binding specificity as described by Sockman et al., (1993), v. 268, p. 22874 or Seiffert et al., (1994), J. Biol. Chem., v. 269, p. 2659.

The second antibody carries a label which may be any suitable directly detectable label or a component of any suitable signal-generating system. Many examples of these are well known from the field of immunoassay.

Labelling of the second antibody with a detectable label or a component of a signal-generating system may be carried out by techniques well known in the art. Examples of labels that can be utilized to render an antibody detectable include radioisotopes, enzymes, fluorescent and

chemiluminescent substances. For example, a radioactive element may be used as a directly detectable label; exemplary radioactive labels include the γ-emitters ^{124}I, ^{125}I, ^{128}I, and ^{131}I. A fluorescent label may also be used as a directly detectable label; for example, suitable fluorophores include coumarins such as umbelliferone, rare earth metal ions, chelates or chelate complexes, fluoresceins, rhodamine and rhodamine derivatives.

Suitable labels also include metal complexes, stable free radicals, vesicles, liposomes, colloidal particles, latex particles, spin labels, biotin/avidin and their derivatives.

Chemiluminescent labels include cyclic diacyl hydrazides, including luminol and isoluminol, acridinium esters and related compounds, pyridopyridazines, dioxeranes and bioluminescent proteins such as luciferases.

Enzyme-linked signal-generating systems may be used, including alkaline phosphatase, amylase, luciferase, catalase, beta-galactosidase, glucose oxidase, glucose-6- phosphate dehydrogenase, hexokinase, horseradish peroxidase, lactamase, urease and malate dehydrogenase. The activity of the enzyme can be detected by measuring absorbency, fluorescence or luminescence intensity after reacting the enzyme with an appropriate substrate. When enzymes are used as a label, the linkage between enzyme and antibody may be achieved by conventional methods such as glutaraldehyde, periodic acid and maleimide methods.

Solid matrices to act as solid supports suitable for immobilizing an antibody include microtitre plates, such as those obtainable from Falcon Plastics, Oxnard, Calif., or, for example, regular ELISA microtitre plates (Immulon II, Dynax, Chantilly, V.A.) and Streptavidin-coated ELISA microtitre plates (Reacti-Bind, Pierce, Rockford, IL, and microtitre strips, such as those obtainable from Dynatech, Alexandria, Va. The wells of the strips or the microtitre plates are made of clear plastic material, preferably polyvinyl chloride or polystyrene. Other solid matrices useful for antibody immobilisation include polystyrene tubes, sticks or paddles of any conveni nt

size, polystyrene beads, polyacrylamide matrices, paramagnetic particles, latex particles or gelatin particles.

Antibodies may be immobilised on a solid support by conventional methods which are well known in the art, for example as described in U.S. Patent No. 5,352,583.

In accordance with a preferred embodiment of the invention, a sample of a biological fluid is contacted with a first antibody which binds selectively to PAI-1 to form a complex, the first antibody being immobilised on a solid support. Sufficient time is allowed to permit binding of the PAI-1 of the sample to the immobilised antibody. The solid support is then washed and contacted with a second antibody which binds selectively to multimeric vitronectin and is labelled with a detectable label or has attached to it a signal-generating system. The label or generated signal bound to the solid support is determined, providing a measure of the PAI-1/multimeric vitronectin complex present in the sample, and hence determining the level of active PAI-1.

In accordance with a more preferred embodiment, the sample is contacted simultaneously with the immobilised first antibody on the solid support and the labelled second antibody.

In a further embodiment, the second antibody may lack a label or signal-generating system component and the solid support-bound second antibody is determined by means of a third antibody bearing a detectable label or signal-generating system component, the third antibody binding selectively to the bound second antibody.

In accordance with a further embodiment, the sample is contacted, either simultaneously or stepwise, with a first antibody which binds selectively to PAI-1 and to which is attached one member of a capture pair and with a labelled second antibody which binds selectively to multimeric vitronectin. The resulting mixture is then contacted with a solid support on which is immobilised the other member of the capture pair. After allowing sufficient time for the labelled PAI-1/multimeric vitronectin complex to bind to the solid support by interaction of the members of the capture pair, the solid support is

washed and the amount of label bound to it is determined, to determine the level of active PAI-1 in the sample. Suitable capture pairs include, for example, biotin/streptavidin. The binding selectivities of the antibodies may be reversed, the first antibody binding selectively to multimeric vitronectin and the labelled second antibody binding selectively to PAI-1.

For example, the first antibody binds selectively to PAI-1 and is biotinylated, while the second antibody, selective for multimeric vitronectin, is labelled with horse radish peroxidase (HRP). The sample/antibody mixture is placed in wells coated with streptavidin. After binding of the complex, the wells are washed and the HRP label is developed by addition of substrate and determined.

In accordance with a further embodiment, active PAI-1 may be determined in a homogeneous assay system, without separation of the PAI-1/multimeric vitronectin/first antibody/second antibody complex; such assays employ a labelled antibody wherein the label displays a detectable change on binding of the antibody, distinguishable from the label attached to unbound antibody. Examples of such assay systems, which can readily be adapted by one of ordinary skill in the art to determination of active PAI-1 by measurement of PAI-1/multimeric vitronectin complex, as described herein, are disclosed in U.S. Patent No. 4,692,404 which employs an enzyme-labelled antibody and wherein the antibody-bound enzyme is hindered from reaction with its substrate on antigen binding of the antibody; U.S. Patent No. 5,070,025; U.S. Patent No. 4,318,707; U.S. Patent No. 5,589,401 and U.S. Patent No. 5,017,009, the contents of all of which are incorporated herein by reference.

In accordance with a further embodiment, the invention provides a kit for determining active PAI-1 in a biological fluid. The kit comprises (a) a first antibody which binds selectively to PAI-1 and (b) a labelled second antibody which binds selectively to multimeric vitronectin or a second antibody which binds selectively to multimeric vitronectin and a labelled third antibody which binds selectively to the second antibody.

In accordance with a further embodiment, the kit comprises (a) a first antibody which binds selectively to multimeric vitronectin and (b) a labelled second antibody which binds selectively to PAI-1 or a second antibody which binds selectively to PAI-1 and a labelled third antibody which binds selectively to the second antibody.

The anti-PAI-1 or anti-multimeric vitronectin first antibody may be immobilised on a solid support.

The kit may also contain a set of calibration standards. The kit may also optionally contain additional reagents such as diluents or buffers which are employed in the methods of the invention and calibration standards.

Examples

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Example 1

Reagents:

Coating buffer (CB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl
Blocking buffer (BB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl 1% hydrolysed casein
Incubation buffer (IB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl 5 mM EDTA 1% hydrolyzed casein 0.025% Tween-20
Washing buffer (WB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl 0.025% Tween-20

ELISA plates (Immulon II, Dynax)
First antibody: monoclonal anti-PAI-1 antibody
Second antibody: HRP-labelled anti-multimeric vitronectin antibody

Human active recombinant PAI-1: prepared as described by Gils et al., (1996), Biochemistry, v. 35, pp. 7474-7481 or obtained commercially (American Diagnostica, Greenwich, CT or Molecular Innovations, Royal Oak, MI).

Calibration standards are prepared as follows:

In making a Vn/PAI-1 complex for the standard, an excess of Vn is utilized in order to ensure that no free active PAI-1 is left unbound. Multimeric vitronectin (mVn) at concentration of 1.3 μm is mixed with human rPAI-1 at a concentration of 0.37 μm and incubated at ambient temperature for 30 minutes. The mixture is then diluted in PAI-1 free plasma (Biopool International, Ventura, CA or American Diagnostica, Greenwich, CT) to concentration of a 200 ng of PAI-1/mL, then serially diluted in PAI-1-free plasma and stored frozen at –70C.

The wells of a regular ELISA microtitre plate (Immulon II) are coated with 100 μl/well of CB containing anti-PAI-1 monoclonal antibody (5-15 μg/ml). Plates are incubated at 4°C for 16 to 18 hours, washed three times with WB, blocked with 200 μl/well BB for 1 hour and washed three times with WB.

50 μl portions of plasma samples or of various concentrations of PAI-1/mVn complex standards (prepared as above: final concentrations of PAI-1 in the PAI-1/mVn complex range from 0 to 100 ng/ml) are added to wells, followed by 50 μl/well HRP-labelled anti-mVn monoclonal antibody (2-5 μg/ml in IB). The plates are incubated at room temperature for 60 minutes with shaking, washed three times with WB and developed with HRP substrate for 15 minutes according to manufacturer's instructions (Sigma, St. Louis, Mo).

The enzyme reaction is terminated by addition of 100 μl/well concentrated sulfuric acid. The intensity of the resulting colour is determined by reading the absorbency at 492 nm in a microtitre plate reader (Automated Plate Reader MR1200, Dynax, Chantilly VA). The concentration of active PAI-1 in a sample is determined by comparison with the calibration curve.

Exampl 2

Reagents are as described in Example 1. The wells of an ELISA

microtitre plate are coated with 100 μL/well of CB containing anti-PAI-1 monoclonal antibody (5-15μg/ml). The plates are incubated at 4°C for 16-18 hours, the wells are washed three times with WB, blocked with 200 μL/well of BB for 1 hour and then washed three times with WB.

50 μL portions of the plasma samples under testing or of the various concentrations of the PAI-1-mVn complex standards (final concentration of PAI-1 in the PAI-1/mVn complex range from 0 to 100 ng/ml) are added to each well followed by 50 μL well of IB. The plates are then incubate at room temperature with shaking for 60 min. and, washed three times with WB.

100μL of HRP-labelled anti-mVn monoclonal antibody (2-5μg/ml) in IB is added to each well, the plates are then incubated at room temperature with shaking for 60 min, washed three times with WB and developed with the HRP substrate for 15 minutes according to the manufacturer's instructions.

The enzyme reaction is terminated by addition of 100 μL/well of concentrated sulfuric acid. The intensity of the resulting colour is determined by reading adsorbancy at 492 nm in the microtitre plate reader. The concentration of active PAI-1 in a sample is determined by comparison with the calibration curve.

Example 3

Reagents are as described in Example 1 except for the second antibody which is biotinylated and an HRP-conjugated Streptavidin detection system is utilized, to measure bound second antibody.

The wells of an ELISA microtitre plate are coated with 100 μL/well of CB containing anti-PAI-1 monoclonal antibody (5-15μg/ml). The plates are incubated at 4°C for 16-18 hours, the wells are washed three times with WB, blocked with 200 μL/well of BB for 1 hour and then washed three times with WB.

50 μL portions of the plasma samples under testing or of the various concentrations of the PAI-1-mVn complex standards (final concentration of PAI-1 in the PAI-1/mVn complex range from 0 to 100ηg/ml) are added to each

well, followed by 50 μL/well of biotinylated anti-mVn antibody in IB, at concentration of between 2-5μg/ml. The plates are then incubated at room temperature with shaking for 60 min. and washed three times with WB.

100 μl of HRP-conjugated Streptavidin is added to each well and incubated for 30 min at room temperature with shaking. The plate is washed three times with WB and then developed with the HRP substrate for 15 minutes according to the manufacturer's instructions.

The enzyme reaction is terminated by addition of 100 μL/well of concentrated sulfuric acid. The intensity of the resulting colour is determined by reading adsorbancy at 492 nm in the microtitre plate reader. The concentration of active PAI-1 in a sample is determined by comparison with the calibration curve.

Example 4

Reagents are as described in Example 1 except that the anti-PAI-1 first antibody is conjugated with biotin and the anti-mVn second antibody is labelled with HRP.

Test tubes are used for performing the immune complex formation and then the immune complex binding and development are performed in the wells of streptavidin-coated ELISA microtitre plates (Reacti-Bind, Pierce, Rockford IL).

Procedure:

50 μL of biotinylated anti-PAI-1 antibody (10-15μg/ml) in IB is added to a test tube, followed by 50 μL of HRP labelled anti-mVn antibody (5-15μg/ml) in IB, and then 100 μL of sample to be tested or of the various concentrations of the PAI-1-mVn complex standards (final concentration of PAI-1 in the PAI-1/mVn complex range from 0 to 100 ng/ml). Test tubes are incubated for 60 minutes at room temperature with shaking. Simultaneously, the wells of a Streptavidin-coated microtitre plate are blocked with 200 μL of BB and washed three times with WB.

100 µL of reaction mixture is transferred from each test tube to a well of the blocked Streptavidin-coated microtitre plate and the plate is incubated for 30 minutes at room temperature with shaking. The plate is washed three times with WB and then developed with the HRP substrate for 15 minutes according to the manufacturer's instructions.

The enzyme reaction is terminated by addition of 100 µL/well of concentrated sulfuric acid. The intensity of the resulting colour is determined by reading adsorbancy at 492 nm in the microtitre plate reader. The concentration of active PAI-1 in the sample is determined by comparison with the calibration curve.

The present invention is not limited to the features of the embodiments described herein, but includes all variations and modifications within the scope of the claims.

We claim:

1. A method for determining active plasminogen activator inhibitor-Type 1 (PAI-1) in a biological fluid, the method comprising the steps of:

(i) providing a sample of a biological fluid; and

(ii) measuring the amount of PAI-1/multimeric vitronectin complex in the sample to determine active PAI-1 in the sample.

2. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample either simultaneously or stepwise with a first antibody which binds selectively to PAI-1 and a labelled second antibody which binds selectively to multimeric vitronectin; and

(b) determining the second antibody bound to the complex to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

3. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample either simultaneously or stepwise with a first antibody which binds selectively to multimeric vitronectin and a labelled second antibody which binds selectively to PAI-1; and

(b) determining the second antibody bound to the complex to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

4. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample either simultaneously or stepwise with a first antibody which binds selectively to PAI-1 and a labelled second antibody which binds selectively to multimeric vitronectin;

(b) separating the PAI-1/multimeric vitronectin/first antibody/second

antibody complex formed in step (a) from the sample; and

(c) determining the second antibody bound to the complex to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

5. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample either simultaneously or stepwise with a first antibody which binds selectively to multimeric vitronectin and a labelled second antibody which binds selectively to PAI-1;

(b) separating the PAI-1/multimeric vitronectin/first antibody/second antibody complex formed in step (a) from the sample; and

(c) determining the second antibody bound to the complex to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

6. The method of claim 1 wherein step (ii) comprises the steps of :

(a) simultaneously contacting the sample with a first antibody which binds selectively to PAI-1, the first antibody being immobilised on a solid support, and with a labelled second antibody which binds selectively to multimeric vitronectin ; and

(b) determining the second antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

7. The method of claim 1 wherein step (ii) comprises the steps of :

(a) contacting the sample with a first antibody which binds selectively to PAI-1, the first antibody being immobilised on a solid support;

(b) contacting the solid support with a labelled second antibody which binds selectively to multimeric vitronectin ; and

(c) determining the second antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in

the sample.

8. The method of claim 1 wherein step (ii) comprises the steps of:

(a) simultaneously contacting the sample with a first antibody which binds selectively to multimeric vitronectin, the first antibody being immobilised on a solid support, and with a labelled second antibody which binds selectively to PAI-1 ; and

(b) determining the second antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

9. The method of claim 1 wherein step (ii) comprises the steps of :

(a) contacting the sample with a first antibody which binds selectively to multimeric vitronectin, the first antibody being immobilised on a solid support;

(b) contacting the solid support with a labelled second antibody which binds selectively to PAI-1; and

(c) determining the second antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

10. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample with a first antibody which binds selectively to PAI-1, the first antibody being immobilised on a solid support;

(b) contacting the solid support with a second antibody which binds selectively to multimeric vitronectin;

(c) contacting the solid support with a labelled third antibody which binds selectively to the second antibody; and

(d) determining the third antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

11. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample with a first antibody which binds selectively to multimeric vitronectin, the first antibody being immobilised on a solid support;

(b) contacting the solid support with a second antibody which binds selectively to PAI-1;

(c) contacting the solid support with a labelled third antibody which binds selectively to the second antibody; and

(d) determining the third antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

12. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample, either simultaneously or stepwise, with a first antibody which binds selectively to PAI-1 and to which is attached one member of a capture pair and with a labelled second antibody which binds selectively to multimeric vitronectin to form a mixture;

(b) contacting the mixture with a solid support on which is immobilised the other member of the capture pair; and

(c) determining the second antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

13. The method of claim 1 wherein step (ii) comprises the steps of:

(a) contacting the sample either simultaneously or stepwise, with a first antibody which binds selectively to multimeric vitronectin and to which is attached one member of a capture pair and with a labelled second antibody which binds selectively to PAI-1 to form a mixture;

(b) contacting the mixture with a solid support on which is

immobilised the other member of the capture pair; and

(c) determining the second antibody bound to the solid support to measure the amount of PAI-1/multimeric vitronectin complex in the sample.

14. The method of any one of claims 1 to 13 wherein the biological fluid is selected from the group consisting of whole blood, plasma, serum, urine, saliva, amniotic fluid, cerebrospinal fluid and a tissue extract.

15. The method of any one of claims 1 to 13 wherein the biological fluid is whole blood, plasma or serum.

16. The method of any one of the preceding claims wherein the second antibody is labelled with a directly detectable label.

17. The method of any one of the preceding claims wherein the second antibody is labelled with a component of a signal-generating system.

18. The method of claim 17 wherein the component is an enzyme selected from the group consisting of alkaline phosphatase, amylase, luciferase, catalase, beta-galactosidase, glucose oxidase, glucose-6-phosphate dehydrogenase, hexokinase, horseradish peroxidase, lactamase, urease and malate dehydrogenase.

19. The method of any one of claims 1 to 15 wherein the second antibody is labelled with a fluorophore.

20. The method of claim 19 wherein the fluorophore is selected from the group consisting of a coumarin, a rare earth metal ion, chelate or chelate complex, a fluorescein, rhodamine and a rhodamine derivative.

21. The method of any one of claims 1 to 15 wherein the second antibody is labelled with a luminescent material.

22. The method of claim 21 wherein the luminescent material is selected from the group consisting of a cyclic diacyl hydrazide, luminol, isoluminol, an acridinium ester, a pyridopyridazine, a dioxerane, a bioluminescent protein and a luciferase.

23. The method of any one of claims 1 to 15 wherein the second antibody is labelled with a label selected from the group consisting of a metal complex, a stable free radical, a vesicle, a liposome, a colloidal particle, a latex particle, a spin label and biotin/avidin.

24. The method of any one of claims 6 to 13 wherein the solid support is selected from the group consisting of an ELISA plate, a polyacrylamide matrix, a polystyrene tube, polystyrene beads, latex particles, paramagnetic particles, acrylic particles and gelatin particles.

25. A kit for determining active PAI-1 in a biological fluid comprising:

(a) a first antibody which binds selectively to PAI-1; and

(b) a labelled second antibody which binds selectively to multimeric vitronectin.

26. A kit for determining active PAI-1 in a biological fluid comprising:

(a) a first antibody which binds selectively to multimeric vitronectin; and;

(b) a labelled second antibody which binds selectively to PAI-1.

27. The kit of claim 25 or 26 wherein said first antibody is immobilised on a solid support.

28. The kit of any one of claims 25 to 27 further comprising a set of calibration standards.

29. A kit for determining active PAI-1 in a biological fluid comprising:

(a) a first antibody which binds selectively to PAI-1;

(b) a second antibody which binds selectively to multimeric vitronectin; and

(c) a labelled third antibody which binds selectively to said second antibody.

30. The kit of claim 29 wherein said first antibody is immobilised on a solid support.

31. The kit of claim 29 or 30 further comprising a set of calibration standards.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 04 January 2001 (04.01.01)	
International application No. PCT/CA00/00464	**Applicant's or agent's file reference** 10189-4/PAR
International filing date (day/month/year) 27 April 2000 (27.04.00)	**Priority date** (day/month/year) 28 April 1999 (28.04.99)
Applicant GAWAD, Yahia et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 November 2000 (21.11.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nestor Santesso
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT REQUEST 10189-4/PAR

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0 0-1	**For receiving Office use only** International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4 0-4-1	**Form - PCT/RO/101 PCT Request** Prepared using	PCT-EASY Version 2.90 (updated 08.03.2000)
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	Canadian Patent Office (RO/CA)
0-7	**Applicant's or agent's file reference**	10189-4/PAR
I	**Title of invention**	METHOD FOR DETERMINING PLASMINOGEN ACTIVATOR INHIBITOR
II II-1 II-2 II-4 II-5 II-6 II-7 II-8 II-9	**Applicant** This person is: Applicant for Name Address: State of nationality State of residence Telephone No. Facsimile No.	 applicant only all designated States except US CARDIOGENICS INC. 208 Evans Avenue Suite 214 Toronto, Ontario M8Z 1J7 Canada CA CA 416-251-2890 416-251-5133
III-1 III-1-1 III-1-2 III-1-4 III-1-5 III-1-6 III-1-7	**Applicant and/or inventor** This person is: Applicant for Name (LAST, First) Address: State of nationality State of residence	 applicant and inventor US only GAWAD, Yahia c/o CardioGenics Inc. 208 Evans Avenue Suite 214 Toronto, Ontario M8Z 1J7 Canada CA CA

PCT REQUEST 10189-4/PAR

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III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	PEKATCH, Tanya
III-2-5	Address:	70 Dixfield Drive Apartment 801 Toronto, Ontario M9C 1J1 Canada
III-2-6	State of nationality	CA
III-2-7	State of residence	CA
IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent
IV-1-1	Name (LAST, First)	RAE, Patricia, A., (Dr.)
IV-1-2	Address:	Sim & McBurney 6th Floor 330 University Avenue Toronto, Ontario M5G 1R7 Canada
IV-1-3	Telephone No.	416-595-1155
IV-1-4	Facsimile No.	416-595-1163
V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY CA CH&LI CN CR CU CZ DE DK DM DZ EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT TZ UA UG US UZ VN YU ZA ZW

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V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	**Exclusion(s) from precautionary designations**	NONE	
VI-1	**Priority claim of earlier national application**		
VI-1-1	Filing date	28 April 1999 (28.04.1999)	
VI-1-2	Number	60/131,339	
VI-1-3	Country	US	
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Check list**	number of sheets	electronic file(s) attached
VIII-1	Request	4	-
VIII-2	Description	19	-
VIII-3	Claims	7	-
VIII-4	Abstract	1	abstract.txt
VIII-5	Drawings	0	-
VIII-7	TOTAL	31	
	Accompanying items	paper document(s) attached	electronic file(s) attached
VIII-8	Fee calculation sheet	✓	-
VIII-16	PCT-EASY diskette	-	diskette
VIII-18	**Figure of the drawings which should accompany the abstract**		
VIII-19	**Language of filing of the international application**	English	
IX-1	**Signature of applicant or agent**		
IX-1-1	Name (LAST, First)	RAE, Patricia, A., (Dr.)	

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/EP

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10-6	Transmittal of search copy delayed until search fee is paid	

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11-1	Date of receipt of the record copy by the International Bureau	

PCT (ANNEX - FEE CALCULATION SHEET)

10189-4/PAR

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(This sheet is not part of and does not count as a sheet of the international application)

0 0-1	**For receiving Office use only** International Application No.				
0-2	Date stamp of the receiving Office				
0-4 0-4-1	**Form - PCT/RO/101 (Annex)** **PCT Fee Calculation Sheet** Prepared using	PCT-EASY Version 2.90 (updated 08.03.2000)			
0-9	**Applicant's or agent's file reference**	10189-4/PAR			
2	**Applicant**	CARDIOGENICS INC., et al.			
12	**Calculation of prescribed fees**		fee amount/multiplier	total amounts (CAD)	
12-1	Transmittal fee T		⇨	200	
12-2	Search fee S		⇨	1,450	
12-3	International fee Basic fee (first 30 sheets) b1		630		
12-4	Remaining sheets	1			
12-5	Additional amount (X)	15			
12-6	Total additional amount b2		15		
12-7	b1 + b2 = B		645		
12-8	Designation fees Number of designations contained in international application	85			
12-9	Number of designation fees payable (maximum 8)	8			
12-10	Amount of designation fee (X)	136			
12-11	Total designation fees D		1,088		
12-12	PCT-EASY fee reduction R		-194		
12-13	Total International fee (B+D-R) I		⇨	1,539	
12-17	**TOTAL FEES PAYABLE (T+S+I+P)**		⇨	3,189	
12-19	**Mode of payment**	cheque			
12-20	**Deposit account instructions** The receiving Office:	Canadian Patent Office (RO/CA)			
12-20-2	is hereby authorized to charge any deficiency or credit any over-payment in the total fees indicated above to my deposit account	✓			
12-20-3	is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account	✓			
12-21	Deposit account No.	00000			
12-22	Date	27 April 2000 (27.04.2000)			

PCT (ANNEX - FEE CALCULATION SHEET) 10189-4/PAR

Original (for **SUBMISSION**) - printed on 27.04.2000 02:11:28 PM

12-23	Name and signature	RAE, Patricia, A., (Dr.)

VALIDATION LOG AND REMARKS

13-2-6	Validation messages Contents	Yellow! The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
		Green? The international application contains no drawings. Please verify.
		Green? Priority 1. The priority document is not enclosed. (The applicant must furnish it within 16 months from the earliest priority date claimed)

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PCT-EASY INFORMATION SHEET

(For applicant use only, DO NOT submit this sheet with the international application)

VALIDATION LOG

	Contents
Yellow!	The power of attorney or a copy of the general power of attorney will need to be furnished unless all applicants sign the request form.
Green?	The international application contains no drawings. Please verify.
Green?	Priority 1. The priority document is not enclosed. (The applicant must furnish it within 16 months from the earliest priority date claimed)

Before submitting the International Application, please carefully verify that:

-the information contained on printed Request form is correct;
-Box IX of the Request form has been signed;
-all elements of the international application as indicated in Box VIII of the Request form have been attached; and,
-the diskette containing the PCT-EASY zip file of the International Application has been enclosed and has been clearly labeled "PCT-EASY", with the applicant's or agent's file reference, and the first applicant's name.

ATTENTION

DO NOT modify any indications on the Request form printout. The attached PCT-EASY application has been locked. If an error or an omission is discovered at this time, you must copy the submitted application as a template and make the change or correction in a new application (using the submitted application as a template). You may create such a template by copying the submitted application from the "Stored Forms" folder to the "New PCT Forms" folder. Open the new (.0WO) file created in the "New PCT Forms" folder, correct the errors and proceed with the submission process again.

NL

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
10189-4/PAR		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/CA 00/ 00464	27/04/2000	28/04/1999
Applicant		
CARDIOGENICS INC. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. --

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SEIFFERT D (REPRINT): "The glycosaminoglycan binding site governs ligand binding to the somatomedin B domain of vitronectin" JOURNAL OF BIOLOGICAL CHEMISTRY, (11 APR 1997) VOL. 272, NO. 15, PP. 9971-9978. PUBLISHER: AMER SOC BIOCHEMISTRY MOLECULAR BIOLOGY INC, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814. ISSN: 0021-9258., XP002149848 DUPONT MERCK RES LABS, EXPT STN, E400-3438, POB 80400, WILMINGTON, DE 19809 (Reprint);SCRIPPS CLIN & RES INST, DEPT VASC BIOL, LA JOLLA, CA 92037 cited in the application page 9972, left-hand column, paragraph 2 - paragraph 3 ---	1-31
X	LAWRENCE ET AL: "Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 12, 21 March 1997 (1997-03-21), pages 7676-7680, XP002149849 cited in the application page 7677, right-hand column, paragraph 2 -page 7678, left-hand column, paragraph 1 ---	1-31
X	LAWRENCE ET AL: "Localization of vitronectin binding domain in plasminogen activator inhibitor-1" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 21, 27 May 1994 (1994-05-27), pages 15223-15228, XP002149850 page 15224, left-hand column, paragraph 4 ---	1-31
X	DECLERCK ET AL: "Purification and characterization of a plasminogen activator inhibitor binding protein from human plasma" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 30, 25 October 1988 (1988-10-25), pages 15454-15461, XP002149851 abstract page 15455, left-hand column, paragraph 3 page 15457, left-hand column, paragraph 2 - paragraph 3; figures 5,6 page 15458, right-hand column, last paragraph -page 15459, left-hand column, paragraph 1; figure 9 --- -/--	1-31

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	KOST ET AL: "Mapping of binding sites for heparin" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 17, 15 June 1992 (1992-06-15), pages 12098-12105, XP002149852 page 12099, left-hand column, paragraph 4 -right-hand column; figure 1	1-31

INTERNATIONAL SEARCH REPORT

[redacted]rmation on patent family members

[redacted]ternational Application No
PCT/CA 00/00464

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9739028 A	23-10-1997	AU 2665397 A	07-11-1997
		CA 2233670 A	23-10-1997
		EP 0850252 A	01-07-1998
		US 6103498 A	15-08-2000
WO 9816643 A	23-04-1998	AU 7431796 A	11-05-1998
		EP 0934410 A	11-08-1999

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/CA 00/00464

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 G01N33/53 G01N33/543 C12Q1/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 39028 A (AMERICAN NAT RED CROSS) 23 October 1997 (1997-10-23) example III ---	1-31
X	WO 98 16643 A (COLEMAN TIMOTHY A ;HUMAN GENOME SCIENCES INC (US); LAWRENCE DANIEL) 23 April 1998 (1998-04-23) page 43, line 6 -page 44, line 15 page 53, line 1 - line 14 --- -/--	1-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2000	08/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Routledge, B

2

Form PCT/ISA/210 (second sheet) (July 1992)

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
RAE, P.
Sim & McBurney
330 University Avenue
6th floor
Toronto, Ontario M5G 1R7
CANADA

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	21.12.2000
Applicant's or agent's file reference: 10189-4/PAR	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00464	27/04/2000	28/04/1999

International Patent Classification (IPC) or both national classification and IPC
G01N33/53

Applicant
CARDIOGENICS INC. et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☒	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 28/08/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Thiele, U

Formalities officer (incl. extension of time limits)
Digiusto, M
Telephone No. +49 89 2399 8162

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-19 as originally filed

Claims, No.:

1-31 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrialapplicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1
Inventive step (IS)	Claims	1-31
Industrial applicability (IA)	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

S ction V

1) Reference is made to the following documents:

D1: WO 97 39028 A
D2: WO 98 16643 A
D3: JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, NO. 15, pages 9971-9978, cited in the application
D4: JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 12, 21 March 1997, pages 7676-7680, cited in the application on page 2
D5: JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 21, 27 May 1994, pages 15223-15228
D6: JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 30, 25 October 1988, pages 15454-15461
D7: JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 17, 15 June 1992, pages 12098-12105

2) The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of claim 1 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

D2 (page 53, lines 1 - 14; page 43, line 6 - p. 44, line 15) anticipates assaying in biological samples for active PAI-1 via its complexes with vitronectin and suggests various detection techniques.

3) Notwithstanding the afore made novelty objection, the subject-matter of claim 1 does not involve an inventive step in view of D1 and D3 - D6 (Art. 33(3), Rule 65(1)(2) PCT).

a) D1 (Example III, page 63 et seq.) reports that it is only the active form of PAI-1 which binds to vitronectin. The assays are done with both native and urea-purified vitronectin. It is evident from page 8 of the instant description that the term "multimeric vitronectin" as used in the present claims means a polymer of monomeric vitronectin that occurs naturally in plasma.

Each of D3 (page 9971, l. col., paragraphs 2 and 3) and D5 (page 15224, l. col., paragraph 4) discloses an assay which detects the binding of only the active forms of PAI-1 to vitronectin multimers. Bound PAI-1 is detected using anti-human PAI-1 IgG.

D4 (page 7677, r. col., paragraph 3 - page 7677, l. col., paragraph 1) pertains to essentially analogous subject-matter.

D6 identifies a PAI-1 binding protein as vitronection and discloses assays for determining active vitronectin.

b) Although D2 - D6 are not related to determining active PAI-1 in a biological sample, it is considered that the skilled person, once the general concept of active PAI-1 / vitronectin complexes is known, would have had motivation and sufficient guidance to apply the teaching disclosed in said documents to an vivo situation, and would thus have arrived with a high expectation of success at the subject-matter of claim 1.

It is generally accepted that the skilled person working in one field (here: detection of complexes from purified components) would regard a means conveniently adopted in a neighbouring field (here: detection of the same complexes in biological samples) as readily usable also in that field, if this transfer of technical knowledge involves nothing out of the ordinary.

2) Dependent claims 2 - 24 do not appear to contain any additional features which, in combination with the feature of the claim(s) to which they refer, involve an inventive step (Art. 33(3) PCT). The said additional features are purely conventional in the technical field concerned.

3) The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of claims 25 - 31 does not involve an inventive step (Rule 65(1)(2) PCT).

The phrase "for determining active PAI-1 [...]" has to be construed as meaning suitable for the indicated purpose (Guidelines, C-III, 4.8).

None of documents D1 - D7 makes explicit mention of a kit comprising the components necessary for immunologically detecting active PAI-1.

However, it is evident that the ensemble of components claimed in claims 25 - 31 automatically comes into existence whenever the known or non-inventive methods of claims 1 - 24 are performed.

4) D7 would appear not to relate to methods for determining active PAI-1 and is considered to merely represent distant state of the art.

Section VII

1) The incorporation of prior art by reference is not allowed as the international application should be self-contained (see further Guidelines, C-II, 4.17). Phrases such as "[...] incorporated herein by reference." to be found e.g. on page 14 contravene said requirement. The same applies to references to non-published patent applications.

2) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1, D2, D5 and D6 is not mentioned in the description, nor are these documents identified therein.

PATENT COOPERATION TREATY

FAX: (416) 595 1163

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

RAE, P.
Sim & McBurney
330 University Avenue
6th floor
Toronto, Ontario M5G 1R7
CANADA

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	05.04.2001
Applicant's or agent's file reference: 10189-4/PAR	**REPLY DUE** **within 1 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00464	27/04/2000	28/04/1999

International Patent Classification (IPC) or both national classification and IPC
G01N33/53

Applicant
CARDIOGENICS INC. et al.

1. This written opinion is the ~~first~~ second drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☐	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☒	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 28/08/2001.

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Thiele, U .

Formalities officer (incl. extension of time limits)
Digiusto, M
Telephone No. +49 89 2399 8162

I. Basis of the opinion

1. With regard to the **elements** of the international application (Replacement *sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*):

Description, pages:

1-19 as originally filed

Claims, No.:

1-31 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

   ☒ the entire international application,

   ☐ claims Nos. ,

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☒ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Section III

The applicant in a letter responsive to the Written Opinion dated 21.12.2000 has presented arguments which make it necessary to reconsider the international preliminary examining authority's, IEA, opinion as previously expressed.

The IEA now considers that the subject-matter of claims 1 - 31 is not supported by the description (Art. 5, 6 PCT). Thus, no meaningful opinion can be given on the novelty and inventiveness of the claimed subject-matter.

1) The applicant, in response (see page 2 of the letter dated 20.03.2000) to the Written Opinion held that the previous literature on the interrelationships between vitronectin, monomeric and multimeric, and PAI-1, active and inactive, was confusing and contradictory, as described in the Background section of the application (see page 2 of the instant description).

The applicant moreover held that D2, which document has been cited in the Written Opinion as prejudicial to the novelty of the present claims, referred only in very general terms to various types of immunoassays in relation to a quite different protein, BAIT protein. There was no detail whatsoever provided specific to the assay of PAI-1. At a completely remote protion of D2, there was a comment that this different protein BAIT protein may interact in the body with protein cofactors analogous to the interaction of PAI-1 with vitronectin.

D6 (letter of 20.03.2001), according to the applicant, reported that plasma vitronectin showed several peaks of PAI-1 activity, as noted at page 15455, Results Section. The main peak of high molecular weight vitronectin contained only 85% of PAI-1 activity. This would suggest, according to the applicant, that measuring the PAI-1 bound to multimeric vitronectin would determine only a portion of the active PAI-1 present in serum.

2) It has to be noted that the present application is devoid of any experimental data whatsoever. There is no experimental proof for the fact that active PAI-1 in a biological fluid can be determined by measuring the amount of PAI-1-multimeric vitronectin complex in the sample.

In the absence of such proof it is considered that the methods of claims 1 - 24 are not supported by the description (Art. 5, 6 PCT). In view of the confusing and contradictory state of the art, and in view of the fact that the present application does not add technical information in terms of concrete data which would go beyond said state of the art, it is not reasonable to predict that the methods claimed have the properties or uses the applicant ascribes to them in the description.

3) The subject-matter of claims 25 - 31 could only be considered as supported by the description when in combination with method claims not objectable.

4) The description does not describe in detail at least one way of carrying out the invention claimed using examples (Rule 5(1)(v) PCT).

No particular ways of determining the PAI-1/multimeric vitronectin complex are detailed. There are merely vague statements and assertions having no technical content as regards anti-multimeric vitronectin antibodies. It is merely speculated that unique epitopes exposed in denatured vitronectin will also be present in the multimeric vitronectin of the active PAI-1/multimeric vitronectin complex (see present description page 9, bottom paragraph). However no such antibodies have been produced or have been publicly available at the relevant application date of the present application.

Thus, it is considered that the skilled person would be unable, on the basis of the information given in the application as filed, to extend the mere speculations presented in the description by using routine methods to the whole of the field claimed, i.e. claims 1 - 31 (Art. 5, 6 PCT).

5) The present invention as claimed in claims 1 - 31 thus merely amounts to the presentation of a scientific theory (Rule 67(1)(i) PCT) and the skilled person would have to exercise inventive skills to carry out, on the basis of the information provided in the application as originally filed, the invention as claimed.

Section VII

The applicant's comments have been noted.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

RAE, P.
Sim & McBurney
330 University Avenue
6th floor
Toronto, Ontario M5G 1R7
CANADA

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	02.07.2001
Applicant's or agent's file reference 10189-4/PAR	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00464	27/04/2000	28/04/1999

Applicant
CARDIOGENICS INC. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Neumann, M Tel.+49 89 2399-7351

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10189-4/PAR		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00464	27/04/2000	28/04/1999
International Patent Classification (IPC) or national classification and IPC G01N33/53		
Applicant CARDIOGENICS INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☐ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/11/2000	02.07.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Thiele, U Telephone No. +49 89 2399 8643

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00464

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-19 as originally filed

Claims, No.:

1-31 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☒ the entire international application.

☐ claims Nos. .

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☒ the claims, or said claims Nos. 1-31 are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Section III

1) The applicant, in response (see page 2 of the letter dated 20.03.2000) to the Written Opinion held that the previous literature on the interrelationships between vitronectin, monomeric and multimeric, and PAI-1, active and inactive, was confusing and contradictory, as described in the Background section of the application (see page 2 of the instant description).

The applicant moreover held that D2 (WO 98 16643 A), which document has been cited in the Written Opinion as prejudicial to the novelty of the present claims, referred only in very general terms to various types of immunoassays in relation to a quite different protein, BAIT protein. There was no detail whatsoever provided specific to the assay of PAI-1. In a completely remote part of D2, there was a comment that this different BAIT protein may interact in the body with protein cofactors analogous to the interaction of PAI-1 with vitronectin.

D6 (JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 30, 25 October 1988, pages 15454-15461), according to the applicant (letter of 20.03.2001), reported that plasma vitronectin showed several peaks of PAI-1 activity, as noted at page 15455, Results Section. The main peak of high molecular weight vitronectin contained only 85% of PAI-1 activity. This would suggest, according to the applicant, that measuring the PAI-1 bound to multimeric vitronectin would determine only a portion of the active PAI-1 present in serum.

2) It has to be noted that the present application is devoid of any experimental data whatsoever. There is no experimental proof for the fact that active PAI-1 in a biological fluid can be determined by measuring the amount of PAI-1-multimeric vitronectin complex in the sample.

In the absence of such proof it is considered that the methods of claims 1 - 24 are not supported by the description (Art. 5, 6 PCT). In view of the confusing and contradictory state of the art, and in view of the fact that the present application does not add technical information in terms of concrete data which would go beyond said state of the art, it is not reasonable to predict that the methods claimed have the properties or uses the applicant ascribes to them in the

description.

3) The subject-matter of claims 25 - 31 could only be considered as supported by the description when in combination with non-objectable method claims.

4) The description does not describe in detail at least one way of carrying out the invention claimed using examples (Rule 5(1)(v) PCT).

No particular ways of determining the PAI-1/multimeric vitronectin complex are detailed. There are merely vague statements and assertions having no technical content as regards anti-multimeric vitronectin antibodies. It is merely speculated that unique epitopes exposed in denatured vitronectin will also be present in the multimeric vitronectin of the active PAI-1/multimeric vitronectin complex (see present description page 9, bottom paragraph). However, no such antibodies have been produced or have been publicly available at the relevant application date of the present application.

Thus, it is considered that the skilled person would be unable, on the basis of the information given in the application as filed, to extend the mere speculations presented in the description by using routine methods to the whole of the field claimed, i.e. claims 1 - 31 (Art. 5, 6 PCT).

5) The present invention as claimed in claims 1 - 31 thus merely amounts to the presentation of a scientific theory (Rule 67(1)(i) PCT) and the skilled person would have to exercise inventive skills to carry out, on the basis of the information provided in the application as originally filed, the invention as claimed.

Section VII

1) Under some national or regional patent systems, the incorporation of prior art by reference is not allowed as the application should be self-contained. Phrases such as "[...] incorporated herein by reference." to be found e.g. on page 14 would then in a later regional or national phase possible contravene said requirement. The

same applies to references to non-published patent applications.

2) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 (WO 97 39028 A), D2 (WO 98 16643 A), D5 (JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 21, 27 May 1994, pages 15223-15228) and D6 (JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 30, 25 October 1988, pages 15454-15461) is not mentioned in the description, nor are these documents identified therein.

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are with the one chosen by the applicant. The full name or two-letter code of that Authority be indicated by the applicant on the line

IPEA/ _____

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: 10189-4/PAR

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/CA00/00464	27 April 2000 (27/04/00)	28 April 1999 (28/04/99)

Title of invention
METHOD FOR DETERMINING PLASMINOGEN ACTIVATOR INHIBITOR

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

CardioGenics Inc.
208 Evans Avenue
Suite 214
Toronto, Ontario
M8Z 1J7
Canada

Telephone No.: (416) 251-2890
Facsimile No.: (416) 251-5133
Teleprinter No.:

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
CA	CA

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

GAWAD, Yahia A.
208 Evans Avenue
Suite 214
Toronto, Ontario
M8Z 1J7
Canada

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
CA	CA

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

PEKATCH, Tanya
70 Dixfield Drive
Apt. #801
Toronto, Ontario
M9C 1J1
Canada

State *(that is, country)* of nationality:	State *(that is, country)* of residence:
CA	CA

☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/CA00/00464

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s) /common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official The address must include postal code and name of country.)*

RAE, Patricia A. (Dr.)
SIM & McBURNEY
6th Floor
330 University Avenue
Toronto, Ontario
M5G 1R7
Canada

Telephone No.: (416) 595-1155

Facsimile No.: (416) 595-1163

Teleprinter No.:

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

☒ the international application as originally filed.

the description ☒ as originally filed
☐ as amended under Article 34

the claims ☒ as originally filed
☐ as amended under Article 19 (together with any accompanying statement)
☐ as amended under Article 34

the drawings ☒ as originally filed
☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examing Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

☒ which is the language in which the international application was filed.
☐ which is the language of a translation furnished for the purposes of international search.
☐ which is the language of publication of the international application.
☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/CA00/00464

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	1 sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ nucleotide and or amino acid sequence listing in computer readable form
6. ☐ other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Patricia A. Rae (Dr.)

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

For International Bureau use only

Demand received from IPEA on:

CHAPTER II

PCT

FEE CALCULATION SHEET

Annex to the Demand for international preliminary examination

International application No.	PCT/CA00/00464
Applicant's or agent's file reference	10189-4/PAR

For International Preliminary Examining Authority use only

Date stamp of the IPEA

Applicant

CardioGenics Inc. et al.

Calculation of prescribed fees

1. Preliminary examination fee	2,998.29	P
2. Handling fee *(Applicants from certain States are entitled to a reduction of 75% of the handling fee. Where the applicant is (or all applicants are) so entitled, the amount to be entered at H is 25% of the handling fee.)*	287.51	H
3. Total of prescribed fees Add the amounts entered at P and H and enter total in the TOTAL box	3,285.80	TOTAL

Mode of Payment

- ☐ authorization to charge deposit account with the IPEA (see below)
- ☐ cheque
- ☐ postal money order
- ☒ bank draft
- ☐ cash
- ☐ revenue stamps
- ☐ coupons
- ☐ other *(specify)*:

Deposit Account Authorization *(this mode of payment may not be available at all IPEAs)*

The IPEA/ _____ ☐ is hereby authorized to charge the total fees indicated above to my deposit account.

☐ *(this check-box may be marked only if the conditions for deposit accounts of the IPEA so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

Deposit Account Number _____ Date *(day/month/year)* _____ Signature _____

10189-4/PAR

283

Serial No.: PCT/CA00/00464

Applicant: CardioGenics Inc. et al.

Title: **METHOD FOR DETERMINING PLASMINOGEN ACTIVATOR INHIBITOR**

International Filing Date: April 27, 2000

Examiner: Thiele, U

Date: March 20, 2001

DELIVERED VIA FACSIMILE (Confirmation by Courier)
(Fax No. 011-49-89-23 99-44 65)

REPLY

International Preliminary Examining Authority
European Patent Office
Erhardstrasse 27
D-80298 München
Germany

Dear Sirs:

This amendment is responsive to the Written Opinion dated December 21, 2000, (21.12.2000) in the above-identified International Application.

The Present Invention

As discussed at page 2 of the specification as filed, of the total non-platelet plasminogen activator inhibitor Type 1 (PAI-1) in circulation in humans, about 60% is active PAI-1 and about 40% is inactive PAI-1. If one wishes to measure the circulating level of active PAI-1, one must exclude the inactive PAI-1.

It is known that PAI-1 binds to the protein vitronectin which occurs in platelets and in circulation; it is also known that vitronectin can exist in monomeric and multimeric forms in platelets. It has been reported that plasma vitronectin is monomeric (Seiffert (1997), J. Biol. Chem., v. 272, p. 9971). The previous literature on the interrelationships between vitronectin, monomeric and multimeric, and PAI-1, active and inactive, is confusing and contradictory, as described in the Background Section of the application, and the Examiner must view the present invention against a background of all of this art and must avoid selecting from this art assisted by hindsight, with the benefit of the teachings of the subject application.

The present application teaches that one can measure the level of active PAI-1 in circulation in a biological fluid such as blood, plasma or serum, by determining the amount of PAI-1 in the fluid which is complexed to multimeric vitronectin.

Novelty

In paragraph 2 of the Written Opinion, the Examiner holds that the subject matter of claim 1 lacks novelty in view of reference D2, International Patent Application WO97/39028 and the Examiner refers specifically to page 43, line 6 to page 44, line 15 and to page 53, lines 1 to 14.

Reference D2 describes the identification of a new protein, expressed in human brain, which is a member of the serpin superfamily, a serine protease inhibitor and shows Tissue-Type Plasminogen Activator Inhibitory Activity. This brain protein is referred to in the reference as BAIT.

The BAIT protein described in reference D2 is not the same protein as PAI-1; it has a different amino acid sequence and a different spectrum of plasminogen activator inhibitory activity.

At pages 43 to 44, it is stated that BAIT protein levels in a biological sample may be assayed "using any art-known method". There follows a series of paragraphs in very general terms referring, inter alia, to various types of immuno-assays. There

is no detail whatsoever provided specific to the assay of BAIT protein or of any Plasminogen Activator Inhibitor.

At page 53, it is noted that PAI-1 binds to the protein vitronectin and that this binding modulates the protease-inhibitory activity of PAI-1. This is also discussed in the background section of the subject application, for example at page 2, lines 15 to 20, where it is indicated that about 60% of the total circulating PAI-1 is bound to vitronectin.

Reference D2 merely uses this previously published finding regarding PAI-1 as a basis for suggesting that, by analogy, there may be proteins which bind to the quite different protein BAIT and modulate the protease-inhibiting activity of BAIT. It is suggested that one could therefore screen compounds to identify those which enhance or inhibit the action of BAIT on proteases.

Reference D2 does not in any way relate its comments on PAI-1/vitronectin binding to any method for assaying either BAIT or PAI-1.

The present invention provides an improved method for determining the level of active PAI-1 in a biological fluid by determining the amount of PAI-1 in the fluid which is complexed to multimeric vitronectin.

Contrary to the Examiner's assertion, reference D2 offers no suggestion assaying of biological samples for the level of active PAI-1 via any complexes with vitronectin. In one portion of the reference, at pages 43 to 44, there are some very general suggestions that a quite different protein, BAIT protein, may be assayed by purely conventional immunoassay techniques. At a completely remote portion of the reference, there is a comment that this different BAIT protein may interact in the body with protein cofactors analogous to the interaction of PAI-1 with vitronectin.

In order to deprive a claim of novelty, the cited reference has to teach each and every aspect of the invention. As noted in the above paragraph, the cited reference teaches no aspect of the claimed invention and therefore does not anticipate claim 1 or

any other claims of this application.

Inventive Step

The Examiner has also rejected claim 1 as lacking inventive step in view of references D1 and D3 to D6.

Reference D1 discloses mutants of the human PAI-1 protein, some of which interact with vitronectin and therefore inhibit activities stimulated by vitronectin, such as vitronectin-dependent cell migration. The Examiner relies on Example 3 of this reference which describes a study of the binding of PAI-1 to both native and urea-treated vitronectin. At page 65, lines 6-9, it is reported that active PAI-1 binds to both denatured vitronectin and native vitronectin (i.e. monomeric vitronectin). It is also reported that latent or inactive PAI-1 binds to both monomeric and multimeric vitronectin, albeit with lower affinity.

Firstly, both the native vitronectin and the urea-purified vitronectin used in these studies were purified proteins. These studies are therefore not reflective of the conditions which apply when one attempts to assay similar proteins in the complex environment of blood or plasma. Furthermore, these studies suggest that active PAI-1 binds to monomeric vitronectin as well as to aggregated forms such as denatured vitronectin and that not all of the PAI-1 bound to denatured vitronectin is active PAI-1. This reference therefore teaches away from the subject invention.

Reference D3 studies the binding of vitronectin to various ligands. The assay system referred to by the Examiner involved the binding of purified PAI-1 in vitro to SMB polypeptides, i.e. an isolated peptide representing one binding domain of vitronectin (not to vitronectin multimers as asserted by the Examiner). This artificial system is again not reflective of conditions in vivo in plasma and does not offer any guidance as to how to measure circulating active PAI-1 in blood or plasma.

Reference D4 examined the binding of both active and latent (inactive) PAI-1 to vitronectin and found that both forms of PAI-

1 bound to both monomeric and denatured vitronectin (see Abstract). Again, this would not suggest that one could measure active PAI-1, and only active PAI-1, by measuring PAI-1 bound only to multimeric vitronectin.

Reference D5 used mutant PAI-1 proteins to identify the domain of the protein responsible for binding to vitronectin. The assay referred to by the Examiner involved coating microtiter plates with purified vitronectin, applying various recombinant PAI-1 proteins to the coated surface and measuring the amount of PAI-1 bound to the plates. The assay therefore makes no distinction between the binding of active and non-active PAI-1 or between PAI-1 binding to monomeric or multimeric vitronectin. It therefore teaches nothing relevant to the method of the subject invention. Contrary to the Examiner's assertion, the described assay does not detect binding of only active PAI-1 to vitronectin multimers.

It is interesting to note that the authors of Reference D5 comment, at page 15223, right column, on the controversy surrounding the interaction of PAI-1 and vitronectin and the conflicting results of various authors.

Reference D6 is an early paper in which the plasma binding protein for PAI-1 was identified as vitronectin. The Examiner asserts that the assay discloses an assay for determining active vitronectin. The reference does not disclose an assay for determining active PAI-1. The reported studies of plasma vitronectin showed several peaks of PAI-1 activity, as noted at page 15455, Results Section. The main peak of high molecular weight vitronectin contained only 85% of PAI-1 activity. This would suggest that measuring the PAI-1 bound to multimeric vitronectin would determine only a portion of the active PAI-1 present in serum. This reference therefore does not teach or suggest measurement of active PAI-1 by measuring PAI-1 bound to multimeric vitronectin.

The Examiner notes that References D2 to D6 do not relate to determining active PAI-1 in a biological sample. In view of the

inconsistencies and contradictions noted above in the teachings of these references, it is respectfully submitted that these teachings would not have suggested to one skilled in the art that in the more complex context of plasma assays, one could determine total active PAI-1 by measuring PAI-1 bound to naturally occurring multimeric vitronectin.

Accordingly, it is respectfully submitted that the cited references, whether considered singly or in combination, do not deprive the claimed invention of inventive step.

With respect to the Examiner's comments in Section VII, the points noted by the Examiner are not objectionable in all countries and the applicant prefers not to amend the application at this time.

Respectfully submitted,

SIM & McBURNEY

Per:
Patricia A. Rae (Dr.)

PAR/tw

ATENT COOPERATION TR[redacted]TY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10189-4/PAR		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA00/00464	27/04/2000	28/04/1999
International Patent Classification (IPC) or national classification and IPC G01N33/53		
Applicant CARDIOGENICS INC. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☐ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/11/2000	02.07.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Thiele, U Telephone No. +49 89 2399 8643

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA00/00464

I. Basis f the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-19 as originally filed

Claims, No.:

1-31 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☒ the entire international application.

   ☐ claims Nos. .

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☒ the claims, or said claims Nos. 1-31 are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Section III

1) The applicant, in response (see page 2 of the letter dated 20.03.2000) to the Written Opinion held that the previous literature on the interrelationships between vitronectin, monomeric and multimeric, and PAI-1, active and inactive, was confusing and contradictory, as described in the Background section of the application (see page 2 of the instant description).

The applicant moreover held that D2 (WO 98 16643 A), which document has been cited in the Written Opinion as prejudicial to the novelty of the present claims, referred only in very general terms to various types of immunoassays in relation to a quite different protein, BAIT protein. There was no detail whatsoever provided specific to the assay of PAI-1. In a completely remote part of D2, there was a comment that this different BAIT protein may interact in the body with protein cofactors analogous to the interaction of PAI-1 with vitronectin.

D6 (JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 30, 25 October 1988, pages 15454-15461), according to the applicant (letter of 20.03.2001), reported that plasma vitronectin showed several peaks of PAI-1 activity, as noted at page 15455, Results Section. The main peak of high molecular weight vitronectin contained only 85% of PAI-1 activity. This would suggest, according to the applicant, that measuring the PAI-1 bound to multimeric vitronectin would determine only a portion of the active PAI-1 present in serum.

2) It has to be noted that the present application is devoid of any experimental data whatsoever. There is no experimental proof for the fact that active PAI-1 in a biological fluid can be determined by measuring the amount of PAI-1-multimeric vitronectin complex in the sample.

In the absence of such proof it is considered that the methods of claims 1 - 24 are not supported by the description (Art. 5, 6 PCT). In view of the confusing and contradictory state of the art, and in view of the fact that the present application does not add technical information in terms of concrete data which would go beyond said state of the art, it is not reasonable to predict that the methods claimed have the properties or uses the applicant ascribes to them in the

description.

3) The subject-matter of claims 25 - 31 could only be considered as supported by the description when in combination with non-objectable method claims.

4) The description does not describe in detail at least one way of carrying out the invention claimed using examples (Rule 5(1)(v) PCT).

No particular ways of determining the PAI-1/multimeric vitronectin complex are detailed. There are merely vague statements and assertions having no technical content as regards anti-multimeric vitronectin antibodies. It is merely speculated that unique epitopes exposed in denatured vitronectin will also be present in the multimeric vitronectin of the active PAI-1/multimeric vitronectin complex (see present description page 9, bottom paragraph). However, no such antibodies have been produced or have been publicly available at the relevant application date of the present application.

Thus, it is considered that the skilled person would be unable, on the basis of the information given in the application as filed, to extend the mere speculations presented in the description by using routine methods to the whole of the field claimed, i.e. claims 1 - 31 (Art. 5, 6 PCT).

5) The present invention as claimed in claims 1 - 31 thus merely amounts to the presentation of a scientific theory (Rule 67(1)(i) PCT) and the skilled person would have to exercise inventive skills to carry out, on the basis of the information provided in the application as originally filed, the invention as claimed.

Section VII

1) Under some national or regional patent systems, the incorporation of prior art by reference is not allowed as the application should be self-contained. Phrases such as "[...] incorporated herein by reference." to be found e.g. on page 14 would then in a later regional or national phase possible contravene said requirement. The

same applies to references to non-published patent applications.

2) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 (WO 97 39028 A), D2 (WO 98 16643 A), D5 (JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 21, 27 May 1994, pages 15223-15228) and D6 (JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 30, 25 October 1988, pages 15454-15461) is not mentioned in the description, nor are these documents identified therein.